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Protection against viral disease in fish following injection of plasmid DNA encoding neutralising single chain antibodies.

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Abstract

A single chain FV antibody gene encoding a recombinant antibody with neutralising activity against the fish rhabdovirus VHSV was inserted under a strong eukaryotic promoter in a plasmid expression vector. Functionality of the construct in terms of expression of neutralising antibodies was verified by transfection experiments in cell culture. Single chain FV antibodies could also be detected in serum from fish, which had received plasmid DNA, by intra muscular injection. Furthermore, such fish were protected when exposed to a lethal dose of infectious virus. The results indicate that injection of plasmid DNA encoding protective antibodies might be a future strategy for immuno-prophylaxis alternatively to passive immunisation by injection of serum-antibodies.

Introduction

Passive immunisation by injection of homologous or heterologous serum-antibodies is routinely used in humans for immunoprophylaxis of people travelling to foreign regions involving risk of exposure to exotic pathogens . In animals a similar strategy may be employed for protection of valuable individuals, but will generally be too expensive for routine veterinary use. Passive immunisation of animals against infectious diseases is thus mostly done on experimental basis with the aim of studying the function of antibodies in vivo and possibly relate the results to in vitro experiments (Bachmann et al. 1997, Lorenzen et al. 1990).

During the recent decade, diverse technologies for production of antibodies by the use of recombinant DNA technology have been developed . The smallest functional recombinant antibody combining the actions of the heavy (H) and light (L) polypeptide chains as in the native molecule has proved to be the single chain variable-fragment construct (single chain FV). The single chain FV antibody is composed of the variable parts of the H and L chains connected by a flexible spacer region (Colcher et al., 1998). Such molecules have been used in various studies including virus neutralisation (Muller et al. 1997), cancer-immunotherapy (Cochet et al. 1998) and recently also in the form of DNA vaccines where plasmids encoding anti-idiotype single chain FV antibodies have proved able to induce an antigen-specific immuneresponse (King et al. 1998). However, direct establishment of protective immunity to infectious diseases by prophylactic treatment with plasmid DNA carrying single chain FV genes encoding protective antibodies has not been described until now.

Here we report that intra muscular injection of a plasmid construct encoding a virus-neutralising single chain FV antibody can mediate in vivo expression of antibodies

which protect the animals against a lethal exposure to virus. The experimental model involves the fish rhabdovirus called viral haemorrhagic septicaemia virus (VHSV) with rainbow trout (*Onchorhyncus mykiss*) as host species (Jensen 1965, Lenoir and De Kinkelin 1975, Lorenzen et al. 1999).

Materials and methods.

Plasmid constructs

Single chain antibody genes were prepared according to the procedure described by McGregor et al. (1994). In short, the variable domains of the immunoglobulin H and L chain genes were cloned from hybridoma cell lines producing monoclonal antibodies to VHSV (Lorenzen et al. in press, Cupit et al., 1998). Associating the H and L chain variable domains generated the single chain antibody (ScAb) genes by a 14 amino acid linker. As a tag to allow specific detection, the human kappa light chain constant domain gene was included in the 3'end of the gene (McGregor et al., 1994). In order to ensure secretion of the ScAb polypeptides in eukaryotic cells, the nucleotide sequence encoding the 20 amino acid signal peptide of rainbow trout transforming growth factor beta (TGF-beta) was added in the 5' end of the gene (Hardie et al., 1998). The gene construct was inserted by blunt-end ligation into the eukaryotic expression vector pCDNA3 (Invitrogen) in the EcoR I site in the polylinker downstream a cytomegalovirus (CMV) promoter (Fig.1). As a negative control in transfection experiments with cell cultures and immunoprotection trials in fish, the pCDNA3 plasmid without insert was used. Plasmid DNA was purified from over-night cultures of E. coli by the use of commercial kits for anion-exchange chromatography as recommended by the supplier (Qiagen). Other molecular biology procedures followed Sambrook et al. (1989). The variable domain genes from two hybridoma cell lines were included: One secreting the non-neutralising monoclonal antibody IP1H3 and another secreting the VHSV- neutralising monoclonal antibody 3F1H10 (Lorenzen et al., 1988, Lorenzen et al., 1990, Olesen et al., 1993, Lorenzen et al., in press). Cloning and sequencing of the variable domain genes has been described earlier (Lorenzen et al., in press). In the case of antibody 3F1H10, two amino acid substitutions were introduced in the H-chain gene (Asn35 to Thr, Lys64 to Thr), since earlier work on antibodies expressed in E. coli had indicated that this would enhance the neutralising ability of the ScAb (Cupit et al. 1998). The ScAb including the variable domains of antibody IP1H3 was called BG5 whereas the ScAb carrying the variable domains of antibody 3F1H10 was called BU1.

Analyses for SCAb-expression in transfected cell cultures.

EPC cells from carp (Fijan et al.) were transfected in wells of 24-well cell culture trays with an anionic transfection reagent (Superfect, Qiagen) as described earlier (Lorenzen et al. 1998). Four to six days after transfection, cell culture supernatants were harvested and analysed for antibody reactivity to VHSV. After removal of supernatants, the cells remaining attached to the bottom of the cell culture wells were fixed in 80% cold acetone and stained by immuno-peroxidase (Lorenzen et al. 1998) using horseradishperoxidase-conjugated rabbit antibody to Human kappa light chain (HRP-Rabbit anti-kappa) (DAKO, Denmark) in order to detect cells expressing ScAb. In a related set-up, different doses of live VHSV was added to wells with cultures of transfected cells 4 days after transfection and the development cytopathogenic effects (CPE) was afterwards recorded.

Injection of plasmid DNA into fish.
Disease free rainbow trout fingerlings, average weight 4.5g, were anaesthetised 0.001% benzokaine and given two 25 µl injections of 20 µg plasmid DNA each, in the epaxial muscles below the dorsal fin. The fish were afterwards kept in groups of approx. 150 individuals in 120-liter tanks supplied with running tap water. The fish were fed ad libitum with commercial fish feed. Mean water temperature was 16 C. Injected plasmid constructs included the pCDNA3 vector without insert, pCDNA3 carrying the ScAb BG5 gene construct (pCDNA3-BG5), and pCDNA3 carrying the ScAb BU1 gene construct (pCDNA-BU1) respectively.

Immunohistochemical analysis for expression of ScAb in injected fish.
Twelve days after injection of plasmid DNA, 10 fish were sampled for each plasmid construct. After termination of the fish a section of muscle tissue was excised from the site of injection. The tissue was fixed in 10% phosphate buffered formalin and analysed by immunohistochemistry following earlier outlined procedures (Lorenzen et al, 1998). Horse radish peroxidase -conjugated rabbit immunoglobulin (Ig) to human kappa light chain (HRP-rabbit anti kappa)(Dako, Denmark) was used for detection of expressed ScAb.

Sampling of plasma from fish.
Blood samples were collected 12 days after injection of plasmid DNA from fish not exposed to VHSV. Due to the small size of the fish, sampling was performed with heparin-treated capillary tubes after cutting off the posterior fin on fully anesthetised fish. The fish were terminated immediately afterwards. After centrifugation at 5000 xg, plasma samples were collected and stored at -80 C until analysed.

Serological examinations for VHSV-reactive ScAbs.
Supernatants from transfected cell cultures as well as plasma samples from DNA-injected fish were examined for anti VHSV reactive ScAbs by a plaque-neutralisation (50% PNT) assay and by an enzyme-linked immunosorbent assay (ELISA)(Lorenzen et al. 1990, Lorenzen et al. in press). The ELISA assay was performed in 96-well microtitre plates coated with purified VHSV. Bound ScAb's were detected with HRP-Rabbit anti-kappa. In order to demonstrate that the virus-neutralising activity detected in the trout plasma was due to the ScAbs produced by the fish and not by trout antibodies, two variants of the 50% PNT assay were also applied. One included parallel examination of the neutralising activity against the virulent VHSV3592B and a neutralisation resistant variant of VHSV 3592B selected by cultivating virus in the presence of the neutralising Mab 3F1A2 which is highly similar to Mab 3F1H10. The other included pre-incubation of the trout plasma with rabbit antibodies to human Ig-kappa or with rabbit antibodies to trout Ig before incubation with virus. The latter assay also involved addition of trout complement as described by Olesen and Jørgensen (1986), in order to detect any virus-neutralising trout Ig activity (Lorenzen and LaPatra, 1999).

DNA-immunoprotection trials in fish
Elleven days after injection of plasmid DNA, groups of fish were exposed to (challenged with) the virulent VHSV DK-3592B isolate by immersion in water

containing 10^5 50% tissue-culture infective doses per ml as described earlier (Lorenzen et al, 1998). Challenge was performed in 8-liter aquaria with 25-31 fish in each. Three replicate aquaria was included for each plasmid construct. Dead fish were afterwards daily recorded and collected. Dead fish from all tanks were analysed virologically for the presence of VHSV. Mean water temperature was 16 C from the time of injection to challenge. Before challenge the fish were adapted to 12 C, and this temperature was kept throughout the 20 day challenge period.

Results

Immunochemical detection of expressed ScAb in cell culture and fish.
After immuno-peroxidase staining using the HRP-rabbit anti human kappa, single cells expressing ScAb could be detected in EPC cell cultures transfected with the plasmid constructs pCDNA3-BG5 and pCDNA3-BU1 (Fig. 2), whereas no positive cells were found in cultures transfected with pCDNA3 without insert. The pCDNA3-BU1 construct gave a higher frequency of positive cells compared to pCDNA3-BG5. Similarly, expression of ScAb could be demonstrated in muscle sections from injected fish (Fig. 3). As in the cell culture experiments, there was a higher frequency of positive cells in fish injected with pCDNA3-BU1 compared to animals injected with pCDNA3-BG5 (not shown). No positive cells were found in fish injected with pCDNA3 without insert.

Interference of ScAbs with propagation of VHSV in cell culture.
When monolayers of EPC cell cultures were inoculated with VHSV four days after transfection, development of cytopathogenic effect (CPE) as a result of multiplication of VHSV was highly different in cultures transfected with pCDNA3-BG5 compared to cell cultures transfected with pCDNA3-BU1. In the latter case only plaques of cells became infected and died and there was no further development of CPE in the 8-day observation period. In contrast, when cultures transfected with pCDNA3 or pCDNA3-BG5 were inoculated, all cells became infected and were destroyed within 3-6 days as in a normal propagation of VHSV in EPC cells (Table 1).

Detection of ScAbs to VHSV in the fish.
When plasma from injected fish was analysed in ELISA for ScAbs recognising VHSV, a strong reaction was found in plasma from fish injected with pCDNA3-BU1. Also plasma from fish injected with pCDNA3-BG5 gave a detectable signal, although approximately 8 fold lower. No reactivity was detected in plasma from fish injected with pCDNA3 without insert. As indicated in Table 2, the limited amounts of plasma available made it necessary to perform the analyses on pools of five individuals.
The 50% PNT analysis was performed on individual plasma samples. All 10 individuals injected with pCDNA3-BU1 neutralised VHSV, whereas no neutralising activity was detected in plasma from fish injected with the two other plasmids (Table 3). When plasma from fish injected with pCDNA3-BU1 was pre-incubated with Rabbit anti human kappa before testing in 50% PNT, the neutralising activity was eliminated, whereas no effect was observed upon pre-incubation with normal rabbit serum or with rabbit serum to trout Ig (Table 4). The neutralising activity of a positive trout serum

control was unaffected by pre-incubation with normal rabbit serum and with Rabbit anti human kappa, but was highly reduced upon pre-incubation with rabbit serum to trout Ig (Table 4). As with the parent monoclonal antibody 3F1H10, plasma samples from fish injected with pCDNA3-BU1 could neutralise the virulent VHSV DK-3592B isolate, but not a neutralisation escape-mutant (not shown).

Infection trial.
When challenged with VHSV DK-3592B 11 days after injection of plasmid DNA, most of the fish injected with pCDNA3-BU1 survived, whereas high mortalities were observed among fish injected with pCDNA3-GB5 or with pCDNA3 (Table 5).

Discussion.
To our knowledge, this is the first report demonstrating establishment of protective immunity to an infectious pathogen in higher vertebrates by administration of genes encoding pathogen specific single chain FV antibodies. As the two antibody gene constructs used in the presented experiments did not give rise to similar levels of ScAb-expressing cells neither in cell culture nor in injected fish, a direct qualitative comparison of the protective pCDNA3-BU1 construct and the non protective pCDNA3-BG5 construct was not possible. However, the protective activity of the pCDNA-BU1 construct fully correlated with the occurrence of neutralising anti-VHSV ScAbs in the plasma of injected fish, and although involvement of non-specific mechanisms cannot be completely excluded, it appears likely that the produced BU1 ScAb has been the major cause of protection following injection of the pCDNA3-BU1 plasmid DNA.
In contrast to DNA-vaccines, including anti-idiotype vaccines, the administration of plasmid borne genes in this case do not involve specific activation of the immunesystem in the individual. The principle is simply that single chain FV antibody polypeptides produced by the cells that take up the administered plasmid will be systemicly distributed by the body fluids and protect the individual if infection with the pathogen occurs. This corresponds to the mechanism of prophylaxis against infectious diseases in humans through administration of antiserum or immunoglobulin from immunised donors or animals, but without side effects such as risk of concomitant transfer of infectious diseases or induction of hypersensitivity following repeated administrations.
In order to avoid that pathogen variability overcome the immunity established by the recombinant monoclonal antibodies, practical use might have to involve administration of genes encoding single chain antibodies to several different epitopes of the pathogen or possibly even a single chain FV antibody gene-expression library towards a given pathogen.
The present experimental set-up with a fish rhabdovirus and its host animal may be of limited practical use, except maybe in case of fish pathogens where no efficient vaccine is available and where the individual fish are highly valuable. Recently, DNA-vaccines inducing expression of the G protein gene have thus proved to be very efficient for protection against VHS in rainbow trout under experimental conditions (Lorenzen et al., 1998). However, if the principle of DNA-immunoprophylaxis can be extended to mammals and to humans in particular, it may come up as valuable tool for transcient protection of individuals, such as travellers, against exposure to pathogens where no efficient vaccines are available. Although single chain FV antibodies have been reported to be of low immunogenecity, an optimal gene construct should probably involve homologous antibody genes (in terms of animal species) if possible. Epitope

specificity can then be modulated by site directed mutagenesis in the variable domain genes at the cloning step. Theoretically, biological effector functions such as complement activation could also be built in at this step. Beside the prophylactic aspect, it may also be assumed that plasmid constructs carrying genes encoding pathogen/disease antigen specific single chain FV antibodies could be of therapeutic use in case certain diseases where the host immunesystem itself is unable to produce antibodies with the necessary activity.

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Table 1. Susceptibility of transfected EPC cell cultures to VHSV

Plasmid construct used for transfection	**Cytopathogenic effect upon inoculation with VHSV***
pCDNA3	Complete destruction of cell layer
pCDNA3-BG5	Complete destruction of cell layer
pCDNA3-BU1	Plaques

*Concentrations of VHSV: 10^2-10^3 TCID-50/ml cellculture medium.

Table 2. Antibody reactivity in fish plasma: ELISA

Fish No.*	Injected plasmid	Reactivity with VHSV (A - 496 nm)	
		Dilution: 1/10	Dilution: 1/80
1-5	pCDNA3	0.0	0.0
6-10		0.0	0.0
11-15	pCDNA3-BG5	0.2	0.0
16-20		0.4	0.1
21-25	pCDNA3-BU1	3.0	1.0
26-30		3.0	1.0

*The plasma samples were analysed in pools of 5 individuals.

Table 3. Antibody reactivity in fish plasma: Neutralisation of VHSV

Fish No.*	Injected plasmid	PNT-titres**
1-10	pCDNA3	<10
11-20	pCDNA3-BG5	<10
21-30	pCDNA3-BU1	160-640

* Plasma samples were analysed individually.
**Titres represent the reciprocal value of plasma dilutions reducing the number of plaques to approx. 50% compared to a control well without antibody/plasma.

Table 4. Effect of preincubation of trout plasma with rabbit antibodies on PNT-titres*.

Fish No.	Injected reagent	PNT-titres		
		Normal rabbit	Rabbit to kappa	Rabbit to trout Ig
21-30 (1 pool)	pCDNA3-BU1	640	<40	320-640
Positive trout serum A7.1	Killed VHSV	>10240	>10240	320

* In order to allow detection of neutralising trout antibodies, trout complement was included as described by Olesen and Jørgensen (1986).

Table 5. Protection against VHS

Injected plasmid	Accumulated mortality 20 days post challenge (mean of triplicate tanks).
pCDNA3	81%
pCDNA3-BG5	86%
pCDNA3-BU1	6%

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Figure legends.

Fig. 1.
Schematic drawing of the pCDNA3 plasmid with a ScAb gene construct inserted downstream the CMV promoter. The ScAb gene construct is further described in the text.

Fig. 2.
Culture of EPC cells transfected with pCDNA3-BU1. Twelve days after transfection, the cells were fixed and stained immunochemically using HRP-rabbit anti human kappa for detection of cells containing ScAb. Dark cells are positive.

Fig. 3.
Histological section of muscle tissue sampled from a fish 12 days after injection of pCDNA3-BU1. The section was stained immunochemically using HRP-rabbit anti human kappa for detection of ScAb. Several cells turned out positive (arrow-heads) along the regenerating needle track (injection site) (arrow).

SAMMENDRAG

Protection against viral disease in fish following injection of plasmid DNA encoding neutralising single chain antibodies.

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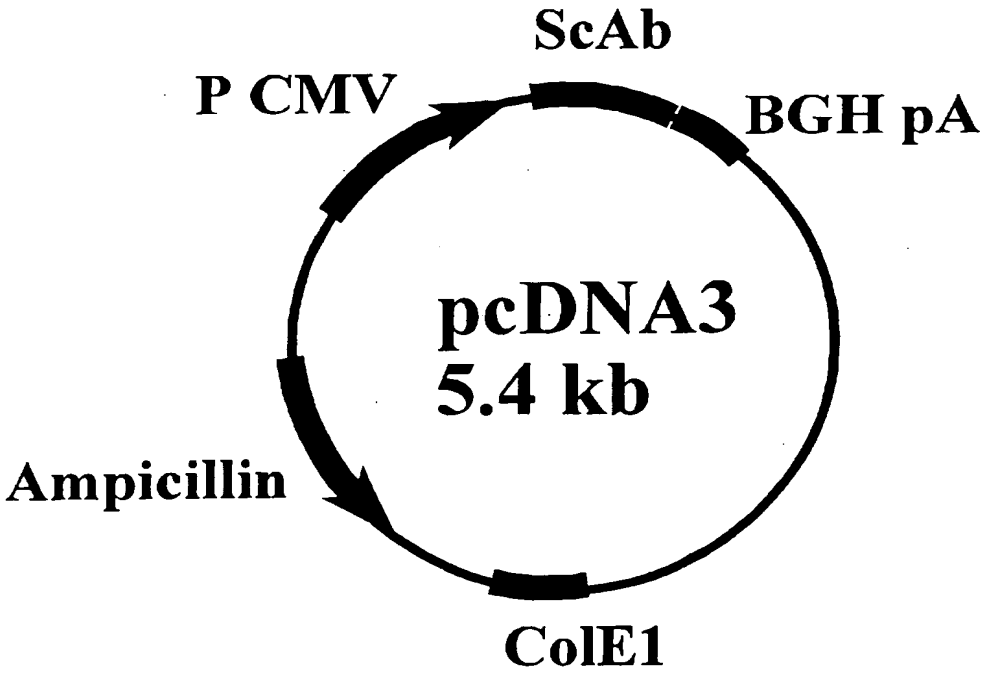
Abstract

A single chain FV antibody gene encoding a recombinant antibody with neutralising activity against the fish rhabdovirus VHSV was inserted under a strong eukaryotic promoter in a plasmid expression vector. Functionality of the construct in terms of expression of neutralising antibodies was verified by transfection experiments in cell culture. Single chain FV antibodies could also be detected in serum from fish, which had received plasmid DNA, by intra muscular injection. Furthermore, such fish were protected when exposed to a lethal dose of infectious virus. The results indicate that injection of plasmid DNA encoding protective antibodies might be a future strategy for immuno-prophylaxsis alternatively to passive immunisation by injection of serum-antibodies.

Fig. 1

DNA plasmid



ScAb gene

Fig 2

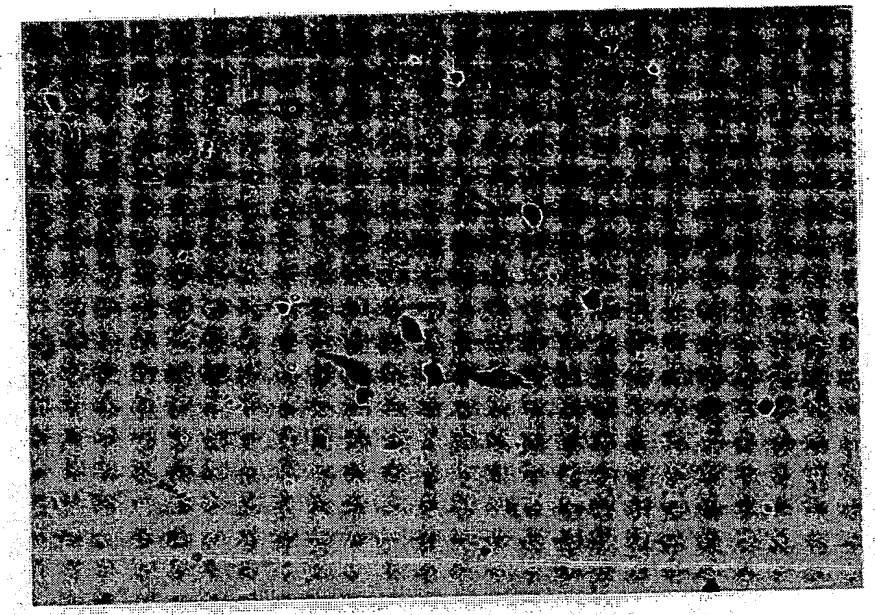


Fig 3